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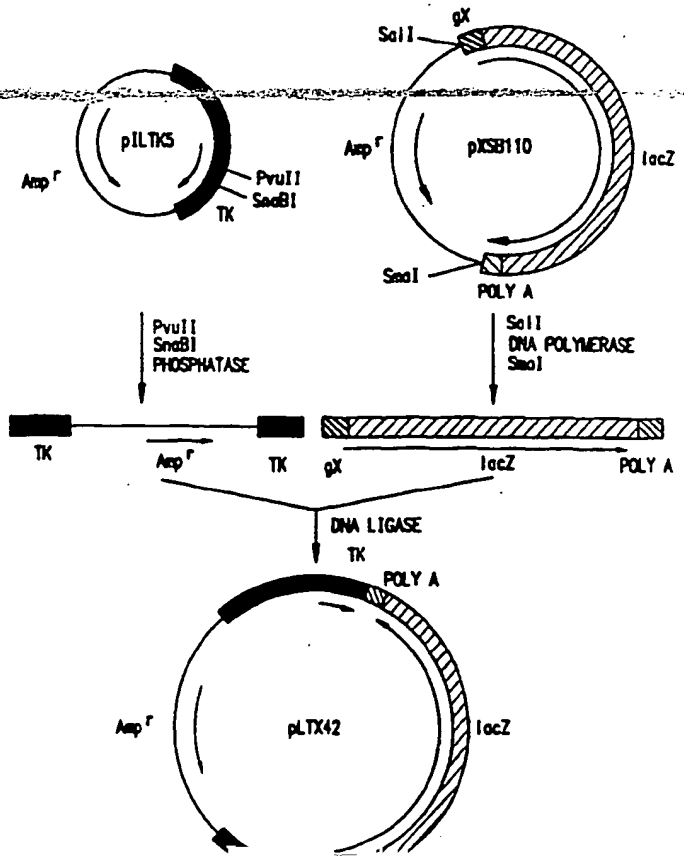
(54) Title: RECOMBINANT INFECTIOUS LARYNGOTRACHEITIS VIRUS AND VACCINE

(57) Abstract

A recombinant avian infectious laryngotracheitis (ILT) virus and avian vaccine containing the recombinant virus in which the virus has foreign DNA inserted into a gene of the virus. The gene contributes to virulence of the virus and insertion of the foreign DNA into the gene lessens or eliminates the virulence of the virus.



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Recombinant Infectious Laryngotracheitis Virus and Vaccine

The present application is a continuation-in-part of U.S. application Serial No. 08/268,683 filed on June 30, 1994.

Background of the Invention

The commercial poultry industry suffers significant financial losses due to respiratory diseases, such as infectious laryngotracheitis (ILT). This prevalent respiratory infection in chickens is caused by the alphaherpesvirus, infectious laryngotracheitis virus (ILTV). Acute infection of poultry by this virus results in reduced egg production and even mortality. Moreover, chickens which apparently recover from the disease, can harbor the virus for various lengths of time. These "carrier" birds are of considerable epizootiological significance, since the latent virus can be reactivated and thus may be responsible for future outbreaks of ILT. Currently, ILTV of varying degrees of pathogenicity are used as vaccines by the poultry industry. However, these strains of ILTV may be capable of reverting to more virulent forms. Thus, there is a need for creating an ILTV vaccine in which the viruses in the vaccine are incapable of reverting to virulent forms.

Summary of the Invention

The present invention fills this need by providing a recombinant avian infectious laryngotracheitis (ILT) virus having foreign DNA inserted into a gene of the virus wherein said gene contributes to virulence of the virus and insertion of the foreign DNA into the gene lessens or eliminates the virulence of the virus. Embodiments of the present invention include recombinant viruses in which the foreign DNA is inserted into the thymidine kinase (TK) gene of the avian infectious laryngotracheitis virus. In specific embodiments of the present invention, the foreign DNA is inserted into the *Pvu*II site, the *Sna*BI site, or replaces a *Pvu*II/*Sna*BI portion of the TK gene. In a

preferred embodiment, the foreign DNA is comprised of a reporter gene, preferably the *E. coli lacZ* gene.

The present invention further provides for a vaccine which comprises an effective immunizing amount of a recombinant ILT virus of poultry of the present invention and a pharmaceutically acceptable carrier.

Brief Description of the Drawings

Figure 1 is a schematic representation of the construction of pILTK5. The relative positions and direction of transcription of the ILTV thymidine kinase (TK) gene and the bacterial β-lactamase (Amp^r) gene are shown.

Figure 2 is a schematic representation of the construction of pXSB1. The relative positions and direction of transcription of the pseudorabies virus glycoprotein X gene promoter (gX) and the bacterial β-lactamase gene (Amp^r) are shown.

Figure 3 is a schematic representation of the construction of pGS2A. The relative positions and direction of transcription of the swinepox virus thymidine kinase (SPV TK) gene, SV40 polyadenylation sequence (poly A), the bacterial chloramphenicol transferase (CAT), *lacZ*, and the β-lactamase (Amp^r) genes are shown. Transcription of the *lac* Zgene is regulated by the vaccinia virus late P11 (VV P11) promoter.

Figure 4 is a schematic representation of the construction of pXSB110. The relative positions and direction of transcription of the swinepox virus thymidine kinase (SPV TK) gene, SV40 polyadenylation sequence (poly A), the bacterial*lacZ*,and the β-lactamase (Amp^r) genes are shown. Transcription of the *lac* Z gene is regulated by either the vaccinia virus late P11 (VV P11) or the pseudorabies virus glycoprotein X gene promoter (gx). Fusion of the gX and *lac* Z genes in pXSB110 is shown at the nucleotide level. The point of ligation is indicated by the vertical dotted line.

Figure 5 is a schematic representation of the construction of pLTX24. The relative positions and direction of transcription of the ILTV

thymidine kinase (TK) gene, SV40 polyadenylation sequence (poly A), the bacterial*lac* Z, and β-lactamase (Amp^r) genes are shown. Transcription of the *lac* Z gene is regulated by the pseudorabies virus glycoprotein X gene promoter (gx).

Figure 6 is a schematic representation of the construction of pLTX36. The relative positions and direction of transcription of the ILTV thymidine kinase (TK) gene, SV40 polyadenylation sequence (poly A), the bacterial *lac*Z, and the β-lactamase (Amp^r) genes are shown. Transcription of the *lac* Z gene is regulated by the pseudorabies virus glycoprotein X gene promoter (gx).

Figure 7 is a schematic representation of the construction of pLTX42. The relative positions and direction of transcription of the ILTV thymidine kinase (TK) gene, SV40 polyadenylation sequence (poly A), the bacterial *lac*Z, and the β-lactamase (Amp^r) genes are shown. Transcription of the *lac* Z gene is regulated by the pseudorabies virus glycoprotein X gene promoter (gx).

Figure 8 is a schematic representation of the construction of pLTX44. The relative positions and direction of transcription of the ILTV thymidine kinase (TK) gene, SV40 polyadenylation sequence (poly A), the bacterial *lac*Z, and the β-lactamase (Amp^r) genes are shown. Transcription of the *lac* Z gene is regulated by the pseudorabies virus glycoprotein X gene promoter (gx).

Figure 9 is a schematic representation of the restriction endonuclease maps of a *Xho*I fragment of the ILTV L608, ILTV-*lac* Z-42, and ILTV-*lac*Z-44 genomes containing the thymidine kinase (TK) gene. The relative positions of the ILTV TK gene, pseudorabies virus glycoprotein X gene promoter (gx), *E. coli lac*Z gene, and SV-40 polyadenylation sequence (poly A) are indicated. All sites recognized by restriction enzymes *Eco*RI, *Pvu*II, *Sal*I, *Sna*BI, and *Xho*I are shown.

Figure 10 is a schematic representation of the restriction endonuclease maps of *Xho*I fragment of the ILTV L608, ILTV- *lac*Z-24 and ILTV-*lac*Z-36 genomes containing the thymidine kinase (TK) gene. The relative positions of the ILTV TK gene, pseudorabies virus glycoprotein X gene promoter (gx), *E. coli lac*Z gene, and SV-40 poly A sequence are

indicated. All sites recognized by restriction enzymes *Eco*RI, *Pvu*II, *Sal*I, *Sna*BI, and *Xho*I are shown.

Detailed Description of the Invention

As stated above, the present invention provides for a recombinant avian infectious laryngotracheitis (ILT) virus having foreign DNA inserted into a gene of the virus wherein said gene contributes to virulence of the virus such that insertion of the foreign DNA into the gene lessens or eliminates the virulence of the virus. Embodiments of the present invention include recombinant viruses in which the foreign DNA is inserted into the thymidine kinase (TK) gene of the avian infectious laryngotracheitis virus. In specific embodiments of the present invention, the foreign DNA is inserted into the *Pvu*II site, the *Sna*BI site, or replaces a *Pvu*II/*Sna*BI portion of the TK gene.

The present invention further provides a recombinant ILT virus of poultry in which the foreign gene which is inserted into the thymidine kinase gene is capable of being expressed in a host cell infected with the recombinant ILT virus such as a polypeptide which the foreign gene encodes.

In one embodiment, the foreign DNA is comprised of a reporter gene, preferably the *E. coli lacZ* gene, which can be expressed when a cell is infected by the virus.

In another embodiment, the polypeptide which the foreign gene expresses is antigenic in the animal into which the recombinant ILT virus of poultry is introduced. The gene encoding for such an antigenic polypeptide can be derived from Newcastle disease virus, infectious bronchitis virus, Mareks' disease virus, infectious anemia virus, infectious bursal disease virus coccidiosis, and pasteurellosis..

If the antigenic polypeptide is encoded by gene from a Mareks' disease virus, the preferred genes are the genes which encode the glycoproteins gB, gA or gD of the Mareks disease virus.

If the antigenic polypeptide is encoded by gene from a Newcastle disease virus, the preferred genes are the genes which encode the New

castle disease virus fusion protein or Newcastle disease virus hemagglutinin-neuraminidase.

If the antigenic polypeptide is encoded by a gene from an infectious bursal disease virus, the preferred gene is the gene which encodes the infectious bursal disease virus VP2 protein.

In order to attenuate ILTV for vaccine use, the virus-encoded thymidine kinase (TK) activity was eliminated by insertional inactivation of the TK gene. A foreign transcriptional unit was inserted into the nucleotide sequence coding for the ILTV TK and the resulting recombinant viruses were identified based on expression of the foreign gene. Initially, plasmids capable of directing this insertion were constructed. Details of this process as well as the methodology used for the creation and identification of TK-negative ILTV are outlined below. Except where indicated, all plasmids were constructed and then verified by restriction endonuclease analysis.

As stated above, the present invention provides for a recombinant avian infectious laryngotracheitis (ILT) virus having foreign DNA inserted into a gene of the virus wherein said gene contributes to virulence of the virus and insertion of the foreign DNA into the gene lessens or eliminates the virulence of the virus. Embodiments of the present invention include recombinant viruses in which the foreign DNA is inserted into the thymidine kinase (TK) gene of the avian infectious laryngotracheitis virus. In specific embodiments of the present invention, the foreign DNA is inserted into the *Pvu*II site, the *Sna*BI site, or replaces a *Pvu*II/*Sna*BI portion of the TK gene. In a preferred embodiment, the foreign DNA is comprised of a reporter gene, preferably the *E. coli lacZ* gene.

The present invention further provides for a vaccine which comprises an effective immunizing amount of a recombinant ILT virus of poultry of the present invention and a suitable carrier. Suitable carriers for the ILT virus of poultry are well known in the art and include proteins, sugars, among others. A suitable carrier is a physiologically balanced culture medium containing one or more stabilizing agents such as stabilized, hydrolyzed proteins, lactose, etc.

An effective immunizing amount of recombinant ILT virus of the present invention is within the range of 10^2 - 10^9 plaque forming units (PFU)/dose.

The present invention also provides for a vector for producing a recombinant ILT virus by inserting foreign DNA into a TK gene of the ILT virus. The vector contains a double-stranded DNA molecule not usually present within the ILT virus of poultry genomic DNA.

EXAMPLES

The present invention can be illustrated by the following, non-limiting Examples. Unless otherwise specified, percentages given below for solids in solid mixtures, liquids in liquids, and solids in liquids are on a wt/wt, vol/vol and wt/vol basis, respectively. Sterile conditions were maintained during cell culture.

Protocols for recombinant ILTV

Example 1

Isolation of LT-Blen® ILTV DNA

Following reconstitution of lyophilized LT-Blen ILTV (obtained from Schering - Plough, Omaha, Nebraska) in a vaccine vial, the virus was inoculated on the, chorioallantoic membranes of developing chicken embryos. After seven days at 37° C in a humidified atmosphere, the chorioallantoic membranes were removed from the eggs. Pock lesions on the membranes were excised and stored at -20° C. Lesions were later ground using a mortar and pestle and the resulting solution was clarified by centrifugation at 5000 rpm at 20° C for 10 minutes (min). The supernatant liquids were saved and the pellets were resuspended in Tris-buffered saline (10 mM'Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM $CaCl_2$, 0.76 mM $MgCl_2$) containing 1% bovine serum albumin, sonicated using a Branson Sonifier (Branson Instruments, Inc.. Danbury, CT), re-ground and then clarified. The combined supernatant liquids were centrifuged at 4° C and 13,500 rpm for 30 min using a Beckman SW 28 rotor. Pellets were resuspended in 3 ml Tris-buffered saline containing

1% bovine serum albumin, sonicated, and then layered on top of a discontinuous sucrose gradient (20%, 25%, 30%, and 40% sucrose sections) in a Beckman SW 28.1 tube. After centrifugation at 4°C and 15,000 rpm for 70 min, virus banding at each of the three sucrose interfaces was separately collected and stored at -20°C. Virus was later concentrated by centrifugation of the samples (diluted approximately 1:5 in Tris-buffered saline) in a SW 28 tube at 4°C and 15,000 rpm for 60 min. The pellets were resuspended in 0.8 ml TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and placed at 4°C. After 18 hr, 15 µl β-mercaptoethanol, 50 µl proteinase K (10 mg/ml) and 200 µl 20% N-lauroyl sarcosinate were added to the resuspended pellets. After a 30 minute incubation at 4°C, 1.4 ml 54% sucrose and 25 µl 20% SDS were added and the lysate was left at 55°C for 3.5 hr. Following the addition of 400 µl 5 M NaCl, the digested nucleocapsids were extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol, once with chloroform and ethanol precipitated at -20°C. Virus DNAs were resuspended in 60 µl TE buffer and stored at -20°C. ILT viruses can also be grown on LMH cell lines as described below.

Example 2

Cloning of the ILTV gene (Figure 1)

A. Creation of pNLTK1

Approximately 5 µg of LT-Blen® ILTV DNA was digested for 4 hr with 10 U of *Xho*I. The resulting 5' overhangs were then "filled in" using 5 U Klenow fragment of DNA polymerase I in the presence of 38 mM Tris-HCl, pH 8.0, 7.7 mM $MgCl_2$, 38 mM NaCl, 100 µM dithiothreitol (DTT), and 125 µM dATP, dCTP, dGTP, and dTTP during a 30 min incubation at 25°C. After the addition of 0.1 vol loading buffer (50% glycerol, 100 mM EDTA, 0.1% bromophenol blue), the fragments were electrophoresed in a 0.75% low melting point agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) at 12 V and 25°C for 16 hr. The DNA pieces were visualized under long wave ultraviolet light after staining with ethidium bromide and those approximately 2 kb in size (as compared to lambda *Hin*dIII and *Eco*RI DNA standards) were excised and stored at -20°C.

Approximately 1 µg pUC9 was digested with 5 U *SmaI for* 3 hr at 30°C. The digested plasmid was extracted once with an equal volume of phenolchloroform-isoamyl alcohol, once with an equal volume of chloroform and then ethanol precipitated. The precipitated plasmid was resuspended in TE buffer and its 5' ends were dephosphorylated using 1 U calf intestine alkaline phosphatase in the presence of 50 mM Tris-HCl, pH 8.5, 0.1 mM EDTA. The reaction was incubated at 37°C for 15 min, at 50°C for 15 min. and then an additional unit of alkaline phosphatase was added and the incubations were repeated. After the addition of 0. 1 vol loading buffer, the plasmid was electrophoresed in a 0.8% low melting point agarose gel at 80 V and 25°C for 2 hr. The band of linearized plasmid was excised.

Agarose gel sections containing either linearized pUC9 or 2 kb ILTV DNA fragments were melted at 70°C and portions were combined and allowed to cool to 37°C. After 15 min, 0.2 vol of 5X DNA ligase buffer and 1 U T4 DNA ligase were added and the reaction was left at 25°C for 18 hr. Prior to being used for transformation, the DNAs were purified by "gene cleaning" (GENE CLEAN®, Bio 101 Inc. LaJolla, CA). Unless otherwise indicated, ligations were performed using the DNA fragments in the molten agarose. A plasmid (pILTK1) with the 2.0 kb *XhoI* fragment containing the ILTV TK gene was identified by restriction enzyme analysis using *Eco*RI and *SalII*. This procedure is summarized and pILTK1 depicted in Figure 1.

B. Creation of pILTK5 (Figure 1)

The 2.0 kb ILTV DNA fragment (containing the TK gene,) was removed from pILTKI and modified in the following manner. Approximately 6 µg of pLTKI was linearized by digestion with 10 U *Bam*HI (2 hr at 37°C) and then 2 µg of the linearized plasmid was digested with either 2, 0.2 or 0.02 U of *Eco*RI (Due to the presence of an *Eco*RI site within the ILTV TK gene, partial digestion must be performed) for 30 min at 37° C. The reactions were then placed at 4° C for 5 min and then the resulting fragments were "blunt ended" using 5 U Klenow fragment of DNA polymerase I in the presence of 38 mM Tris-HCl, pH 8.0, 7.7 mM $MgCl_2$, 77 mM NaCl, 100 µM DTT, and 125 µM

dATP, dCTP, dGTP, and dTTP during a 30 min incubation at 25° C. After the addition of 0.1 vol loading buffer, the fragments were electrophoresed in a 0.8% low melting point agarose gel at 10 V and 25° C for 17 hr. The two reactions, using either 0.2 or 0.02U *Eco*RI, generated the desired 2.0 kb fragment which was gene cleaned in TE buffer.

For ligation with the modified 2.0 kb ILTV DNA fragment (containing the TK gene), approximately 3 µg pGEM3 was digested with 20 U *Hin*dIII for 2.5 hr at 37° C and then the termini of the linearized plasmids were "filled in" using 5 U Klenow fragment of DNA polymerase I in the presence of 38 mM Tris-HCl, pH 8.0, 7.7 mM $MgCl_2$, 38 mM NaCl, 100 µM DTT, and 125 µM dATP, dCTP, dGTP, and dTTP during a 30 min incubation at 25° C. The plasmid was then gene cleaned, digested with 18 U *Pvu*II for 2.5 hr at 37° C, and then gene cleaned again. The 5' ends of the plasmid were dephosphorylated using calf intestine alkaline phosphatase. After the addition of 0.1 vol loading buffer, the plasmid was electrophoresed in a 0.7% low melting point agarose gel at 85 V and 25° C for 2 hr. The desired band of linearized plasmid (2.76 kb) was removed and ligated with the modified 2.0 kb ILTV DNA fragment (containing the TK gene). The resulting plasmid was designated pILTK5. Ligation of the "filled in" *Bam*HI end of the modified 2.0 kb ILTV DNA fragment, excised from pILTK1, with the 'filled in" *Pvu*II end of the modified pGEM3 regenerates the *Bam*HI site which is unique, even when the foreign transcriptional unit is inserted into pILTK5. See Figure 1. This unique site is used to linearize all the plasmids for transfection.

Example 3

Generation of foreign transcriptional unit cassette

A. Creation of pXSB1 (Figure 2)

Pseudorabies (Rice strain) virus DNA was isolated from infected Crandall feline kidney (CRFK) cells by the same procedure used for isolation of ILTV DNA. When approximately 100% of the monolayer exhibited a cytopathic effect (CPE), the cells were pelleted at 2000 rpm at 20° C for 5 min. The cell pellets were resuspended in 20 ml isotonic buffer (10 mM Tris, pH 8.0, 150 NaCl, 5 mM EDTA), re-pelleted at 2000

rpm and 20° C for 5 min, and then resuspended in 9 ml hypotonic buffer (10 mM Tris, pH 8.0, 10 mM KCl, 5 mM EDTA). After sitting on ice for 10 min, 1 ml 10% TRITON X-100® and 25 µl β-mercaptoethanol were added to the resuspended cells. After an additional 10 min on ice, cell nuclei were removed by centrifugation of the cell lysates at 2000 rpm and 4° C for 5 min. The supernatant liquid was then placed in a 30 ml Nalgene Teflon FEP tube and centrifuged at 11,000 rpm and 4° C for 120 min in a Beckman JA-20 rotor. The pelleted cores were resuspended in 0.8 ml TE buffer. Following the addition of 15 µl β-mercaptoethanol, 50 µl proteinase K (10 mg/ml) and 200 µl 20% N-lauroyl sarcosinate, the mixture was placed at 4° C for 30 min. Then, 1.4 ml 54% sucrose and 25 µl 20% SDS were added and the lysate was left at 55° C for approximately 16 hr. After the addition of 400 µl 5 M NaCl, the digested nucleocapsids were, extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol, once with chloroform and ethanol precipitated at -20° C. Pseudorabies virus DNA was resuspended in TE buffer, and stored at -20° C.

Approximately 4 µg of pseudorabies virus DNA was digested with 10 U of *Bam*HI for 4 hr at 37° C. After the addition of 0.1 vol loading buffer, the DNA fragments were electrophoresed in a 0.85% low melting point agarose gel at 12 V and 25° C for 17 hr. The 3.94 kb *Bam*HI-10 fragment (containing the pseudorabies virus gX promoter) was removed.

Approximately 3 µg of pUC18 was digested with 10 U *Bam*HI for 3 hr at 37° C and then gene cleaned into 8 µl TE. The 5' ends of the plasmid were dephosphorylated using calf intestine alkaline phosphatase. After the addition of 0.1 vol loading buffer, the plasmid was electrophoresed in a 0.8% low melting point agarose gel at 12 V and 25° C for 17 hr. The band of linearized plasmid was excised and ligated with the *Bam*HI-10 fragment of the pseudorabies virus genome. The resulting plasmid was designated pB105. (See Figure 2.)

Based on an analysis of the sequence immediately upstream of the gX gene [Van Zijl *et al.*, *J. Gen. Virol.* 71:1747-1755, (1990)], a 430 bp *Bam*HI-*Sal*I fragment derived from the *Bam*HI-10 fragment of the pseudorabies virus genome should contain the entire gX promoter. However, *Bam*HI and *Sal*I digestion of pB105 produces three fragments, approximately 430 bp in size. To circumvent this problem, approximately 10 µg of pB105 was digested with 20 U *Bam*HI and *Xho*I

for 2 hr at 37° C. After the addition of 0.1 vol loading buffer, the fragments were electrophoresed in a 0.8% low melting point agarose gel at 70 V and 25° C for 2 hr. A unique 560 bp fragment was excised and concentrated by gene cleaning. After digestion with 10 U *Sal*I for 3 hr at 37° C, the fragments were electrophoresed in a 1% low melting point agarose gel at 70 V and 25° C for 2 hr. The unique 430 bp fragment (containing the pseudorabies virus gX promoter) was removed.

Approximately 2 µg of pGEM3 were digested with 20 U *Sal*I for 2 hr at 37° C, gene cleaned, and then digested with 8 U *Bam*HI. After the addition of 0. 1 vol loading buffer, the linearized plasmid was electrophoresed in a 1% low melting point agarose gel at 70 V and 25° C for 2 hr. The linearized pGEM3 was ligated with the 430 bp *Bam*HI-*Sal*aI fragment to produce pXSB1. (See Figure 2.)

B. Creation of pGS2A (Figure 3)

A portion of the *Escherichia coli lacZ* gene fused to the SV40 polyadenylation signal sequence was obtained by digestion of approximately 1µg pCAL4 (obtained from Dr. Wagner, University of California at Irvine, Irvine, CA) with 5 U *Cla*I and 5 U *Bam*HI for 2 hr at 37° C. After the addition of 0.1 vol loading buffer, the fragments were electrophoresed in a 0.7% low melting point agarose gel at 45 V and 25° C for 30 min and then at 80 V and 25° C for 2 hr. A 2.65 kb fragment, containing the 2.24 kb 3' terminus of the *lacZ* gene and a 385 bp fragment of SV40 DNA with a polyadenylation signal sequence, was excised from the gel. (See Figure 3.)

The 5' terminal portion of the *lacZ* gene was obtained from pGS108. (Figure 3) This plasmid contains the *lacZ* gene (except for the first eight codons) bounded by *Bam*HI sites and transcriptionally regulated by the vaccinia virus P11 promoter. This transcriptional unit originated in pSC8 (obtained from Dr. Moss, National Institutes of Health, Betheseda, MD) which was subsequently modified to contain an *Xba*I site immediately downstream of the *lacZ* gene pVBX5; [Schnitzlein and Tripathy, *Animal Biotechnology 1*: 161 - 174. (1990)]. This modification enabled the excision of the intact transcriptional unit by *Xba*I digestion of pVBX5 (Note: There already existed a *Xba*I site immediately upstream of the vaccinia virus P11 promoter in pSC8).

The excised DNA was inserted into a central locus of the swinepox virus TK gene which was contained in a pGEM3-type plasmid to create pGS108.

Approximately three-fourths of the *lac*Z gene was removed from pGS108 in the following manner. First, approximately 3 µg of pGS 108 were linearized by digestion with 15 U *Cla*I for 1.5 hr at 37°C. Then, 1µg of the linearized plasmid was digested with either 1, 0.5, or 0.25 U *Bam*HI for 45 min at 37° C. After the addition of 1 µl loading buffer, the fragments were electrophoresed in a 0.7% low melting point agarose gel at 45 V and 25° C for 30 min and then at 80 V and 25° C for 2 hr. A 5.1 kb fragment, corresponding to pGS108 lacking the 3' terminal 2.24 kb of the *lac*Z gene, was removed from the gel. This 5.1 kb fragment was ligated with the 2.63 kb fragment obtained from pCAL4 to produce pGS2A. (See Figure 3.)

C. Creation of pXSB110 (Figure 4)

Transcription of the *lac*Z gene was placed under the regulation of the pseudorabies virus gX gene promoter by inserting the *lac*Z gene downstream of the gX gene promoter in pXSB 1. For this purpose, approximately 2 µg of pXSB1 was digested with *Bam*HI for 2.5 hr at 37° C and then the termini of the linearized plasmid were "blunt ended" using 5 U Klenow fragment of DNA polymerase I in the presence of 38 mM Tris-HCl, pH 8.0, 7.7 mM $MgCl_2$, 77 mM NaCl, 100 µM DTT, and 125 µM dATP, dCTP. dGTP, and dTTP during a 30 min incubation at 25° C. After gene cleaning, the 5' ends of the plasmid were dephosphorylated using calf intestine alkaline phosphatase. After the addition of 0.1 vol loading buffer, the fragments were electrophoresed in a 0.8% low melting point gel at 50 V and 25° C for 3.5 hr. The linearized pXSB1 was removed from the gel.

The *lac*Z gene-SV40 polyadenylation signal sequence fusion was excised from approximately 1 µg of pGS2A by digestion with 8 U *Bam*HI for 2.5 hr at 37° C and then the resulting fragments were "blunt ended" using 5 U Klenow fragment of DNA polymerase I in the presence of 38 mM Tris-HCl, pH 8.0, 7.7 mM $MgCl_2$, 77 mM NaCl, 100 µM DTT, and 125 µM dATP, dCTP, dGTP, and dTTP during a 30 min incubation at 25° C. After the addition of 0.1 vol µl loading buffer, the fragments were

electrophoresed in a 0.8% low melting point agarose gel at 50 V and 25° C for 3.5 hr. The 3.45 kb fragment was removed from the gel and ligated with the linearized pXSB1. The plasmid containing the *lacZ* gene in the proper orientation relative to the gX gene promoter was designated pXSB110. (See Figure 4.)

Production of plasmids.

To create the insertion plasmids, pILTK5 was first linearized with either*Pvu*II, *Sna*BI or a combination of *Pvu*II and *Sna*BI and then ligated with the foreign transcriptional unit. This insert consisted of the intact pseudorabies virus gX gene promoter- *E. coli lacZ* gene-SV40 polyadenylation signal sequence and was obtained as a 3.88 kb fragment following *Sal*I *and Sma*lI digestion of pXSB 110.

Example 4

A. Creation of pLTX24 (Figure 5)

Approximately 3 µg of pILTK5 (Figure 1) was digested with 20 µg *Pvu*II for 2 hr at 37° C. After gene cleaning, the 5' ends of the plasmid were dephosphorylated using calf intestine alkaline phosphatase. After the addition of 0.1 vol loading buffer, the modified plasmid was electrophoresed in a 0.75% low melting point agarose gel at 40 V and 25° C for 15 min and then at 70 V for 2 hr. Linearized pILTK5 was removed from the gel.

Approximately 10 µg of pXSB110 was first digested with 30 U *Sal*I for 2.5 hr at 37° C and then gene cleaned. After digestion with 16 U *Sma*I for 2.5 hr at 37° C, the 5' overhangs (due to *Sal*I digestion) of the fragments were "filled in" using 5 U Klenow fragment of DNA polymerase I in the presence of 15 mM Tris-HCl, pH 7.4, 3.8 mM $MgCl_2$, 38 mM KCl, 100 µM DTT, and 125 µM dATP, dCTP, dGTP, and dTTP during a 30 min incubation at ambient temperature. After the addition of 0.1 vol loading buffer, the fragments were electrophoresed in a 0.75% low melting point agarose gel at 40 V and 25° C for 15 min and then at 70 V for 2 hr. The 3.88 kb fragment was removed from the gel. Prior to ligation, *Pvu*II-linearized pILTK5 and the 3.88 kb fragment were gene cleaned. A plasmid having the foreign gene transcriptional unit

inserted into the *Pvu*II site of pILTK5 was designated pLTX24. See Figures 5 and 10.

B. Creation of pLTX36 (Figure 6)

Approximately 3 µg of pILTK5 (Figure 1) was digested with 20 µg *Sna*BI for 2 hr at 37 C. After gene cleaning, the 5' ends of the plasmid were dephosphorylated using calf intestine alkaline phosphatase. After the addition of 0.1 vol loading buffer, the modified plasmid was electrophoresed in a 0.75% low melting point agarose gel at 40 V and 25° C for 15 min and then at 70 V for 2 hr. Linearized pILTK5 was removed from the gel.

Approximately 10 µg of pXSB110 was first digested with 30 U *Sal*I for 2.5 hr at 37° C and then gene cleaned. After digestion with 16 U *Sma*I for 2.5 hr at 37° C, the 5' overhangs (due to *Sal*I digestion) of the fragments were "filled in" using 5 U Klenow fragment of DNA polymerase I in the presence of 15 mM Tris-HCl, pH 7.4, 3.8 mM $MgCl_2$, 38 mM KCl, 100 µM DTT, and 125 µM dATP, dCTP, dGTP, and dTTP during a 30 min. incubation at ambient temperature. After the addition of 0.1 vol loading buffer, the fragments were electrophoresed in a 0.75% low melting point agarose gel at 40 V and 25° C for 15 min and then at 70 V for 2 hr. The 3.88 kb fragment was removed from the gel. Prior to ligation, *Sna*BI-linearized pILTK5 and the 3.88 kb fragment were gene cleaned. A plasmid having the foreign transcriptional unit inserted into the *Sna*BI site of pILTK5 was designated pLTX36. See Figures 6 and 10.

C. Creation of pLTX42 (Figure 7)

Approximately 2 µg pILTK5 was digested with 10 U *Pvu*II and 10 U *Sna*BI for 4 hr at 37° C. The digested plasmid was extracted once with an equal volume of phenol-chloroform-isoamyl alcohol, once with an equal volume of chloroform and then ethanol precipitated. The precipitated plasmid was resuspended in TE buffer and dephosphorylated using calf intestine alkaline phosphatase. After the addition of 0.1 vol loading buffer, the fragments were electrophoresed in

a 0.8% low melting point agarose gel at 20 V and 25° C for 20 min and then at 80 V for 2 hr. The linearized pLT5 was removed from the gel.

Approximately 3 µg of pXSB110 was first digested with 16 U *Sal*I for 4 hr at 37° C and then the 5' overhangs were "filled in" using 5 U Klenow fragment of DNA polymerase I in the presence of 77 mM Tris-HCl, pH 7.6, 7.7 mM $MgCl_2$, 115 mM NaCl, 100 µM DTT, and 125 µM dATP, dCTP, dGTP, and dTTP during a 30 min incubation at ambient temperature. The modified plasmid was extracted once with an equal volume of phenolchloroform-isoamyl alcohol, once with an equal volume of chloroform and then ethanol precipitated. The precipitated plasmid was resuspended in TE buffer and digested with 20 U *Sma*I for 3 hr at 30° C. After the addition of 0.1 vol loading buffer, the fragments were electrophoresed in a 0.8% low melting point agarose gel at 40 V and 25° C for 20 min and then at 80 V for 2.5 hr. The 3.88 kb fragment was removed from the gel and ligated with *Pvu*II- and *Sna*BI-digested pILTK5. A plasmid having the foreign transcriptional unit in the opposite orientation relative to the ILTV TK, gene was designated pILTV-*lac*Z-42. See Figures 7 and 9.

D. Creation of pLTX44 (Figure 8)

Approximately 2 µg pILTK5 was digested with 10 U *Pvu* II and 10 U *Sna*BI for 4 hr at 37° C. The digested plasmid was extracted once with an equal volume, of phenol-chloroform-isoamyl alcohol, once with an equal volume of chloroform and then ethanol precipitated. The precipitated plasmid was resuspended in TE buffer and dephosphorylated using calf intestine alkaline phosphatase. After the addition of 0. 1 vol loading buffer, the fragments were electrophoresed in a 0.8% low melting point agarose gel at 20 V and 25° C for 20 min and then at 80 V for 2 hr. The linearized pILT5 was removed from the gel

Approximately 3 µg of pXSB110 was first digested with 16 U *Sal*I for 4 hr at 37° C and then the 5' overhangs were "filled in" using 5 U Klenow fragment of DNA polymerase I in the presence of 77 mM Tris-HCl, pH 7.6, 7.7 mM $MgCl_2$, 115 mM NaCl, 100 µM DTT, and 125 µM dATP, dCTP, dGTP, and dTTP during a 30 min. incubation at ambient temperature. The modified plasmid was extracted once with an equal volume of phenolchloroform-isoamyl alcohol, once with an equal

volume of chloroform and then ethanol precipitated. The precipitated plasmid was resuspended in TE buffer and digested with 20 U *Sma*I for 3 hr at 30° C. After the addition of 0.1 vol loading buffer, the fragments were electrophoresed in a 0.8% low melting point agarose gel at 40 V and 25° C for 20 min and then at 80 V for 2.5 hr. The 3.88 kb fragment was removed from the gel and ligated with *Pvu*II- and *Sna*BI-digested pILTK5. A plasmid having the foreign transcriptional unit in the same orientation relative to the ILTV TK gene was designated pILTV-*lac*Z-44. See Figures 8 and 9.

Example 5

Production of recombinant ILTV

Recombinant ILTV were generated using either intact ILTV (ILTV-*lac*Z-36, ILTV-*lac*Z-42, ILTV-*lac*Z-44) or viral nucleocapsids (ILTV-*lac*Z-24). In either case, prior to transfection, the insertion plasmids were linearized by digestion with *Bam*HI. Approximately 20 µg of plasmid were digested with 40 units of *Bam*HI at 37° C for 2 hr.

Note, as indicated earlier, the *Bam*HI site is unique in all insertion plasmids. Since this site is located at a juncture between the plasmid backbone and the inserted ILTV DNA, linearization of the plasmid at this point does not alter the homologous viral DNA required for insertion of the foreign transcriptional unit into the ILTV genome via recombination with the intracellular, replicating viral genomes. Moreover, the linear form of the plasmid may enhance the production of recombinant viruses, since such modified viruses were not obtained using supercoiled plasmids. The digested plasmids were extracted once with an equal volume of phenol-chloroform-isoamyl alcohol, once with an equal volume of chloroform and then ethanol precipitated for 3.5 hr at -20° C. The linearized plasmids were resuspended in TE buffer at a concentration of approximately 0.5 µg/µl and stored at -20' C until used.

ILTV-*lac*Z-24 and ILTV-*lac* Z-36 have the foreign *lac*Z transcriptional unit inserted into their thymidine kinase gene. ILTV *lac*Z-42 and ILTV -*lac*Z -44 have a 258 bp deletion in their thymidine kinase gene which has been replaced by the foreign *lac*Z transcriptional unit.

A. Creation of ILTV-*lac*Z-24

1). Generation of nucleocapsids.

LMH cells in a 150-cc tissue culture flask (Corning), infected with ILTV (estimated to be approximately 500,000 PFU) five days earlier, were pelleted at 2000 rpm and 20° C for 5 min. The cell pellet was resuspended in 5 ml isotonic buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA), re-pelleted and then resuspended in 1.8 ml hypotonic buffer (10 mM Tris, pH 8.0, 10 mM KCl, 5 mM EDTA). After sitting on ice for 10 min, 200 µl 10% TRITON X-100 and 5 µl β-mercaptoethanol were added to the resuspended cells. After an additional 10 min on ice, cell nuclei were removed by centrifugation of the cell lysate at 2000 rpm and 4° C for 5 min. The resulting supernatant liquid was layered over 5 ml of 15% sucrose in hypotonic buffer in a NALGENE TEFLON FEP tube and centrifuged at 4° C and 11,000 rpm for 75 min in a Beckman JA-20 rotor. The pelleted nucleocapsids were resuspended in 0.5 ml Waymouth's medium containing 10 µg/ml gentamicin.

2). Transfection.

A transfection solution was prepared by slowly adding 30 µl 2 X BES-buffered solution [50 mM N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 280 mM NaCl and 1.5 mM Na_2HPO_4, pH 6.95] to 30 µl 0.25 mM $CaCl_2$ containing 2 µg of linearized pLTX24. The resulting mixture was left at ambient conditions for 30 min. Then 50 µl of the transfection solution was added to 0.5 ml of Waymouth's medium supplemented with 1% fetal bovine serum (FBS), 10 µg/ml gentamicin, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B already overlaying an LMH cell monolayer in a 12-well tissue culture plate. Immediately following this addition, 50 µl of ILTV nucleocapsids were added to the medium. The monolayer was gently rocked for approximately 15 min at ambient conditions and placed at 37° C in a humidified atmosphere of 3% CO_2. After 6 hr, the monolayer was washed twice with 1 ml of Waymouth's medium supplemented with 10 µg/ml gentamicin, overlaid with 1.5 ml of Waymouth's medium supplemented with 1% FBS, 10 µg/ml gentamicin, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25

µg/ml amphotericin B and returned to 37° C in a humidified atmosphere of 3% CO_2. After 5 days, the transfected cells were frozen at -80° C until assayed for recombinants.

Note viral nucleocapsids were only used in the creation of ILTV-*lac*Z-24. For transfections involving pLTX36, pLTX42, and pLTX44, cells were infected with intact ILTV.

B. Creation of ILTV-*lac* Z-36

A monolayer of LMH cells in a 12-well plate (CoStar) was infected with 250 µl of ILTV inoculum (approximately 15,000 PFU) for 1 hour at ambient conditions and then overlaid with an additional 750 µl of Waymouth's medium supplemented with 1% FBS, 10 µg/ml gentamicin, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. During this interval, 50 µl of 2 X BES-buffered solution was slowly added with mixing to 50 µl 0.25 mM $CaCl_2$ containing 2 µg of linearized pLTX36. The resulting solution was left at ambient conditions for 30 min and then added to the medium overlaying the infected cells. The monolayer was gently rocked for approximately 15 min at ambient conditions and then placed at 37° C in a humidified atmosphere of 3% CO_2. After approximately 16 hr, the monolayer was washed twice with 1 ml of Waymouth's medium supplemented with 10 µg/ml gentamicin, overlaid with 1.5 ml of Waymouth's medium supplemented with 1% FBS, 10 µg/ml gentamicin, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B and returned to 37° C in a humidified atmosphere of 3% CO_2. After 7 days, the transfected cells were frozen at -80° C until assayed for recombinants. ILTV-*lac*Z--36 has been deposited pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville Maryland, U.S.A.

C. Creation of ILTV -*lac* Z-42

A monolayer of LMH cells in a 12-well plate was infected with approximately 20,000 PFU of ILTV for 1 hour at ambient conditions and

then the inoculum was replaced with 500 µl of Waymouth's medium supplemented with 1% FBS, 10 µg/ml gentamicin, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. During this interval, 30 µl of 2 X BES-buffered solution was slowly added with mixing to 30 µl 0.25 mM $CaCl_2$ containing 2 µg of linearized pLTX42. The resulting solution was left at ambient condition for 30 min and then 50 µl of it was added to the medium overlaying the infected cells. The monolayer was gently rocked for approximately 15 min at ambient condition and then placed at 37° C in a humidified atmosphere of 3% CO_2. After 5.5 hr, the monolayer was washed twice with 1 ml of Waymouth's medium supplemented with 10 µg/ml gentamicin, overlaid with 1.5 ml of Waymouth's medium supplemented with 1% FBS, 10 µg/ml gentamicin, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B and returned to 37° C in a humidified atmosphere of 3% CO_2. After 4 days, the transfected cells were frozen at -80° C until assayed for recombinants.

D. Creation of ILTV-*lac*Z44.

A monolayer of LMH cells in a 12-well plate was infected with approximately 20,000 PFU of ILTV for 1 hour at ambient conditions and then the inoculum was replaced with 500 µl of Waymouth's medium supplemented with 1% FBS, 10 µg/ml gentamicin, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. During this interval, 30 µl of 2 X HBS-buffered solution (50 mM HEPES, pH 7.1, 280 mM NaCl, 1.5 mM Na_2HPO_4) was slowly added with mixing to 30 µl 0.25 mM $CaCl_2$ containing 2 µg of linearized pLTX44. The resulting solution was left at ambient conditions for 30 min and then 50 µl of it was added to the medium overlaying the infected cells. The monolayer was gently rocked for approximately 15 min at ambient conditions and then placed at 37° C in a humidified atmosphere of 3% CO_2. After 5.5 hr, the monolayer was washed twice with 1 ml of Waymouth's medium supplemented with 10 µg/ml gentamicin, overlaid with 1.5 ml of Waymouth's medium supplemented with 1% FBS, 10 µg/ml gentamicin, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B and returned to 37° C in a humidified atmosphere of 3% CO_2. After 4 days, the transfected cells were frozen at -80° C until assayed for recombinants.

E. Screen for recombinant ILTV

Recombinant virus was identified based on its ability to express the *lacZ* gene and thus produce β-galactosidase (*lacZ* gene product). Monolayers of LMH cells in 60- and 100-mm tissue culture plates were infected with the progeny from the transfections. At 4-6 days post infection, the monolayers were overlaid with either 4 (60 mm plate) or 10 (100 mm plate) ml of Waymouth's medium supplemented with 1% FBS, 10 µg/ml gentamicin, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B and containing 0.4% agarose (Ultra Pure DNA Grade; Bio-Rad Laboratories, Richmond, CA). After allowing approximately one hour for the overlays to harden at 25° C, the monolayers were returned to 37° C and a humidified atmosphere of 3% CO_2. On the following day, a second agarose overlay (50% in volume relative to that of the first overlay) containing 300 µg/ml Bluo-gal (halogenated indolyl-β-D-galactoside; GMCO-BRL) was applied. Generally within 16 hr at ambient conditions, cells infected by the recombinant ILTV would turn blue due to hydrolysis of the Bluo-gal substrate by β-galactosidase. Such "blue" plaques were picked using a Pasteur pipette, placed into approximately 1 ml of Waymouth's medium supplemented with 1% FBS, 10 µg/ml gentamicin, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B and stored at -80° C. The selected viruses were plaque-purified by this procedure until only blue plaques were detected in two consecutive rounds of infection. The recombinant viruses were then routinely passaged in LMH cells.

The initial ILT virus used to obtain the initial ILT viral DNA can also be grown on an avian hepatocellular carcinoma cell line, such as the LMH cell line. The LMH cell line is typically grown as cells attached to plastic or glass surfaces in a single monolayer of cells. The cell line can be grown in any suitable medium such as, for example, Waymouth's Medium with 10% fetal calf serum (FCS), CELLGRO® (Mediatech Inc.) with 1-5% FCS, DME-F12 (Sigma Inc.) with 10% FCS, Eagles MEM (Gibco Inc.) with 10% FCS, Medium 199 (Gibco, Inc.) with tryptose phosphate broth and 10% FCS, and Medium 1640 with 10% FCS.

The carcinoma cells are passaged every 5 to 7 days using a 1:5 or 1:10 expansion ratio. The resulting cell monolayers are not allowed to

reach a high density before passage since they become difficult to trypsinize which would result in the subsequent formation of poor monolayers. In passing the cells, the monolayers are drained of medium and then rinsed briefly with a solution containing trypsin, preferably a mixture of trypsin and ethylene diamine tetraacetic acid (EDTA). The cell monolayer allowed is allowed to disperse and the individual cells are then diluted in growth medium to allow for a 1:5 or 1:10 seeding of new flasks. The new cell cultures are then incubated at 37° C in a CO_2 incubator.

Example 6

An avian virus can be grown on the avian hepatocellular carcinoma cells by inoculating the cells with the virus. To inoculate the cell line with an avian virus, confluent or nearly confluent monolayers of cells are drained of medium and the virus inoculum added to the monolayer. The inoculum is allowed to adsorb for about an hour at 37°C and then fresh medium is added and the cultures returned to the incubator. Virus-inoculated cultures are incubated until monolayers show maximum cytopathic effect (CPE). For example the ILT virus requires 1 to 3 days incubation depending on the titer of virus in the inoculum.

The LMH cell line has been deposited pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, U.S.A under ATCC Accession No. CRL 11597.

Example 7

Verification of the TK^- phenotype of the ILTV Recombinants

Attempts to demonstrate the expected TK^- phenotype of the recombinant by directly assaying for enzymatic activity yielded ambiguous results due to the presence of cellular TK. Therefore, a variety of thymine analogs, including 5-(2-bromovinyl)-2'-deoxyuridine and thymine 1-β-D-arabino-furanoside, were examined for their ability

to inhibit virus replication without a commitant loss in cell viability. Among these potential inhibitors, only FMAU [1-(2-fluoro-2-deoxy-β-D-arabinofuranoxyl)-5-methyluracil] [Watanabe, K. *et al.*, Nucleosides. 110. Synthesis and antiherpes acitivity of some 2'-fluoro-2'-deoxy-furanosylpyrimidine nucleosides. *J. Med Chem* 22: 21-24 (1979)] fit the criteria. At a concentration of 9.7 μM, the production of the parental ILTV was reduced by at least almost four orders of magnitude (Table 1). However, the presence of FMAU had very little effect on the replication of the recombinant ILTV. Moreover, the comparable yields of parental and recombinant viruses from infected cells in the absence of FMAU demonstrated that the viral-encoded TK was not essential for virus replication in cell culture.

Monolayers of LMH cells in 12-well plates were infected with approximately 2000 PFU of virus (parent or recombinant). After absorption at ambient temperature for two hours, the inocula were removed. The monolayers were then washed twice and overlaid with Waymouth's medium containing 1% fetal bovine serum, and 9.7 nmoles/ml of the thymine analog, 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-methyluracil (FMAU). At five days post-infection, the cells were frozen at -80°C until assayed for the presence of infectious virus.

TABLE 1

Effect of FMAU on the Replication of ILTV[1]

Virus	FMAU[2]	Virus yield, PFU/ml[3]
	-	5.6×10^5
ILTV L608	+	6.8×10^1
ILTV-*lac*Z-24	-	9.1×10^5
	+	6.8×10^5
ILTV-*lac*Z-36	-	9.0×10^5
	+	8.1×10^5
ILTV L608	-	1.6×10^6
	+	7.4×10^1
ILTV-*lac*Z-42	-	2.4×10^6
	+	8.8×10^5
ILTV-*lac*Z-44	-	3.5×10^5
	+	5.6×10^5

[1] Monolayers of LMH cells in 12-well plates were infected with approximately 2000 PFU of ILTV L608. After absorption at ambient temperature for two hours, the inocula were removed. The monolayers were then washed twice and placed at 37°C. At five days post-infection, the cells were frozen at -80°C until assayed for the presence of infectious virus by titering on LMH monolayers.

[2] When required, 9.7 μM of the thymine analog, FMAU [1-(2-fluro-2-deoxy-β-D-arabinofuranosyl)-5-methyluracil] was included in the medium overlaying the infected monolayers.

[3] Virus yields represent the average of duplicate plaque assays.

Example 8

Safety Testing of Recombinant Virus

Four recombinant laryngotraceitis (LT) viruses were tested for safety. Results summarized in Table 2 show relatively mild respiratory reaction following intratracheal (IT) inoculation. The reactions for

ILTV-*lac*Z-24 were slightly more severe than for ILTV-*lac*Z-36, ILTV-*lac*Z-42, or ILTV-*lac*Z-44. LT-IVAX™ (Stain P2012) was also tested and showed reactions somewhere between ILTV-*lac*Z-24 and the three milder strains. By contrast, the parent L6 strain produced several fold more sever reactions including death in 6 out of 15 birds. The results with ILTV-*lac*Z-24 and ILTV-*lac*Z-36 were similar to those obtained when these two viruses were tested in previous work.

Table 2

Safety Testing of Recombinant Larynogotracheitis Virus in Chickens

ILTV strain	Dosage[f]	Day 2[a]	Day 3[a]	Day 4[a]	Index	Results[d]
*lac*Z-24	4.8	9 (3)[b]	11 (4)	7 (1)	0.8	10/10
*lac*Z-36	4.6	3	4	3	0.2	10/10
lac-Z-42	4.5	4	7 (1)	3	0.3	10/10
*lac*Z-44	4.8	5 (1)	6	2	0.3	10/10
LT-IVAX	4.1	4 (1)	10 (2)	9 (5)	0.6	10/10
TRA-VAX	4.3	15 (15)	15 (15)	15 (15)[c]	2.3	ND[e]
Controls						1/10

a. Respiratory Reaction, Days post-vaccination, No. of total reactions, including mild, moderate and sever.

b. ( ) indicates number of moderate and severe reactions.

c. 6 deaths occurred.

d. Challenge results Number of Chickens protected/Total inoculated.

e. ND = Not determined.

f. Log_{10} $TCID_{50}$/0.2ml

Example 9

The ILTV *lac*Z-42 strain was tested for the Mininmum Protective Dose (MPD_{90}) by regular eyedrop vaccination. This strain was selected for testing because it grew extremely well in LMH cells. Challenge resulted in 2 birds in 10 being protected. This was the highest dosage tested since originally this dosage given intratracheally protected 100%. The reason for this difference is not known but it may reflect differing susceptibility of tracheal *vs* ocular routes of vaccination.

Example 10

The laryngotracheits virus strains ILTV *lac*Z-24 and ILTV *lac*Z-36 were evaluated for safety in 4-week chickens by intratracheal inoculation. Included for comparison were known mild LT-IVAX™ and known severe TRAVAX™.

The results as shown in Table 3 indicate that the ILTV-*lac*Z-24 strain is of reduced virulence when it was compared to the parent TRAVAX™ at equivalent titer. Nonetheless, it is of borderline safety as 2 birds out of 10 died from a reaction to the vaccination. The ILTV *lac*Z-24 when given at reduced titer showed less reaction as expected with LT viruses. Birds which received LT-IVAX™ showed only mild reaction. Challenge of the ILTV-*lac*Z-24 and LT-IVAX-vaccinated birds showed 100% protection for each virus. The TRAVAX™ survivors were not challenged.

The results in Table 4 show that the ILTV-*lac*Z-36 strain produced relatively mild reaction, more than likely because of the low titer LMH stock used for testing. The titer of the virus could not be improved by embryo passage. Nonetheless, this virus appeared efficacious since 13 of

15 inoculated birds were protected against challenge. The estimated virus dosage for this ILTV-*lac*Z-36 stock was only 2.6 logs per bird.

Table 3

Safety and Immunogenicity of Gene Deleted ILTV-*lac*Z-24 in 4-Week-Old Leghorn Chickens Inoculated by Intratracheal Route[a]
4 Days Post-Vaccination

LT Virus	Titer	Neg.[b]	Mild[b]	Mod.[b]	Severe (b)	Dead[b]	Results (c)
*lac*Z-24[f]	4.5/ml	5	2	2	1	0	10/10
*lac*Z-24[g]	6.5/ml	2	2	2	2	2	8/8
LT-IVAX	6.5 ml	8	2	0	0	0	10/10
TRA-VAX	6.5/ml	1	0	0	3	6	ND[d]
Con.[e]							9/10

a Birds inoculated intratracheally with 0.2 ml of virus and then challenged at 18 days by standard methods.

b. Respiratory Reaction 4 days post-vaccination.

c. Number of Protected chickens after challenge/total number of chickens.

d. ND = Not Determined

e. Non-vaccinated Controls.

f. Obtained from a stock of recombinant viruses grown on LMH cells.

g. Obtained from a stock of recombinant viruses grown by chicken embryo passage.

Table 4

Safety and Immunogenicity of Gene Deleted ILTV-*lac*Z-36 in 4-Week-Old Leghorn Chickens Inoculated by Intratracheal Route [a]

LT virus	Titer	Neg.[b]	Mild[b]	Mod.[b]	Severe (b)	Dead	Result[c]
*lac*Z-36	3.6/ml	13	2	0	0	0	13/15
LT-IVAX	6.5/ml	10	4	1	0	0	15/15
TRA-VAX	6.5/ml	2	5	0	3	6	ND[d]
Con[e]							0/10

a Birds inoculated intratracheally with 0.2 ml of virus and then challenged at 18 days by standard methods.

b. Respiratory Reaction 4 days post-vaccination.

c. Number of Protected chickens after challenge/total number of chickens.

d. ND = Not Determined

e. Non-vaccinated Controls.

While the present invention has been described in conjunction with the specific embodiments set forth above, many alternatives, modifications and variations thereof will be apparent to those of ordinary skill in the art. All such alternatives, modifications and variations are intended to fall within the spirit and scope of the present invention.

WHAT IS CLAIMED IS:

1. A recombinant avian infectious laryngotracheitis (ILT) virus wherein the virus has foreign DNA inserted into a gene of the virus, wherein said gene contributes to virulence of the virus and insertion of the foreign DNA into the gene lessens or eliminates the virulence of the virus.

2. The recombinant ILT virus of claim 1 wherein the gene into the foreign DNA is inserted is a thymidine kinase (TK) gene of the ILT virus.

3. The recombinant ILT virus of claim 2 wherein the foreign DNA is inserted into a restriction site of TK gene selected from the group consisting of a *Pvu*II site, a *Sna*BI site, and a *Pvu*II/*Sna*BI site of the TK gene.

4. The recombinant ILT virus of claim 1 wherein the foreign DNA contains a reporter gene.

5. The recombinant ILT virus of claim 4 wherein the reporter gene is an *E. coli lacZ* gene.

6. The recombinant ILT virus of claim 1 wherein the foreign DNA encodes a polypeptide which produces an antigenic response in poultry and wherein the foreign DNA is derived from an avian pathogen.

7. The recombinant ILT virus of claim 6 wherein the avian pathogen is selected from the group consisting of Newcastle disease virus, infectious

bronchitis virus, Mareks disease virus, infectious anemia virus, infectious bursal disease virus, coccidia, and pasteurella.

8. A vaccine for avian infectious laryngotracheitis comprised of a recombinant avian infectious laryngotracheitis (ILT) virus wherein the virus has foreign DNA inserted into a gene of the virus, wherein said gene contributes to virulence of the virus and insertion of the foreign DNA into the gene lessens or eliminates the virulence of the virus.

9. The vaccine of claim 8 wherein the gene into the foreign DNA is inserted is a TK gene of the ILT virus.

10. The vaccine of claim 9 wherein the foreign DNA is inserted into a restriction site of TK gene selected from the group consisting of a *Pvu*II site, a *Sna*BI site, and a *Pvu*II/*Sna*BI site of the TK gene.

11. The vaccine of claim 8 wherein the foreign DNA contains a reporter gene.

12. The vaccine of claim 9 wherein the reporter gene is an *E. coli lacZ* gene.

13. The vaccine of claim 8 wherein the foreign DNA encodes a polypeptide which produces an antigenic response in poultry and wherein the foreign DNA is derived from an avian pathogen.

14. The vaccine of claim 13 wherein the avian pathogen is selected from the group consisting of Newcastle disease virus, infectious bronchitis virus, Mareks disease virus, infectious anemia virus, infectious bursal disease virus, coccidia and pasteurella

15. A vector for producing a recombinant ILT virus by inserting foreign DNA into a TK gene of the ILT virus, wherein the vector contains DNA foreign to the ILT virus.

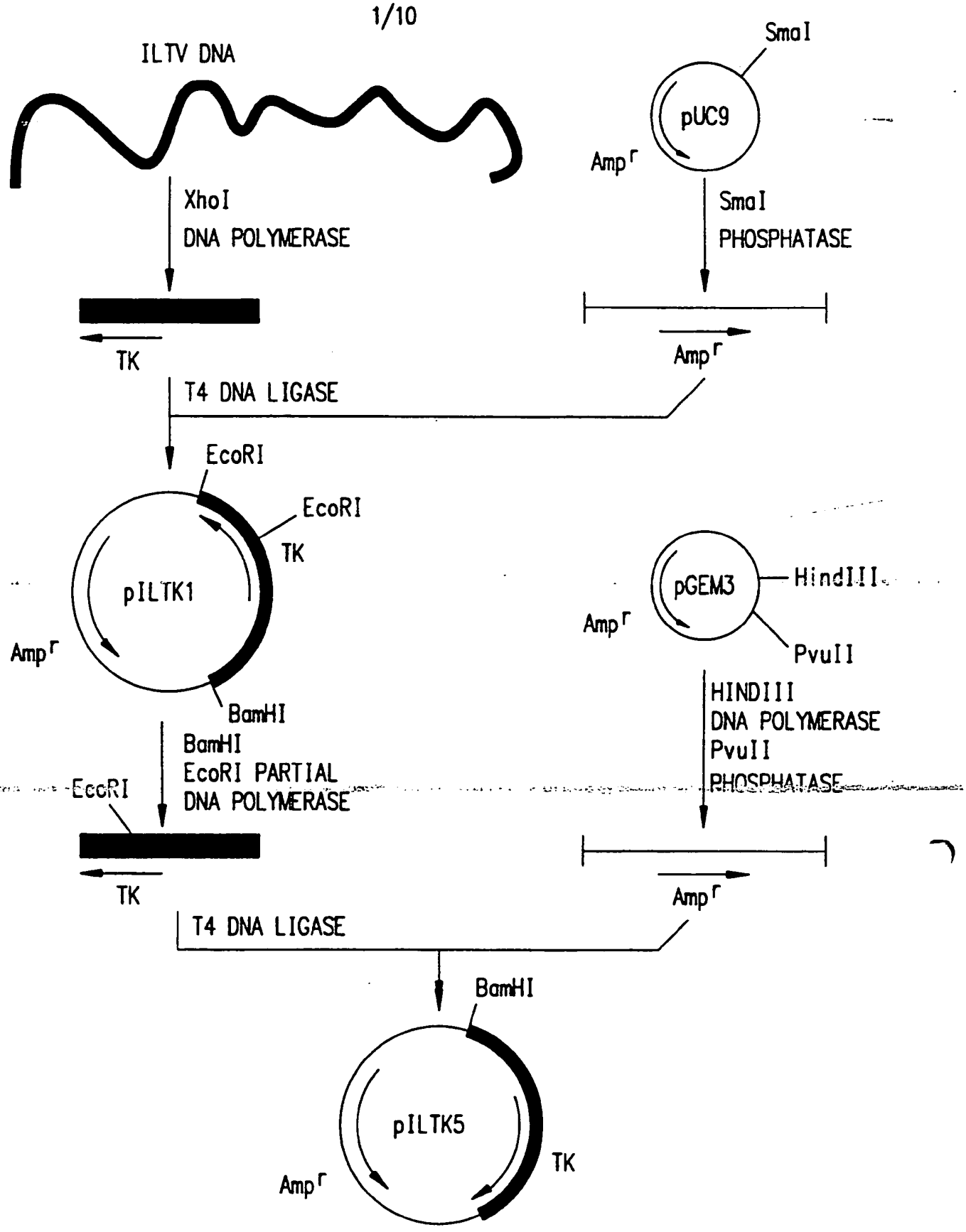
16. The vector of claim 15 wherein the foreign DNA is inserted into a restriction site of the TK gene selected from the group consisting of a *Pvu*II site, a *Sna*BI site, and a *Pvu*II/*Sna*BI site of the TK gene.

17. The vector of claim 15 wherein the foreign DNA contains a reporter gene.

18. The vector of claim 17 wherein the reporter gene is an *E. coli lacZ* gene.

19. The vector of claim 15 wherein the foreign DNA encodes a polypeptide which produces an antigenic response in poultry and wherein the foreign DNA is derived from an avian pathogen.

20. The vector of claim 19 wherein the avian pathogen is selected from the group consisting of Newcastle disease virus, infectious bronchitis virus, Mareks disease virus, infectious anemia virus, infectious bursal disease virus coccidia, and pasteurella.


ILTV DNA
XhoI
DNA POLYMERASE
TK
SmaI
pUC9
Amp r
SmaI
PHOSPHATASE
Amp r
T4 DNA LIGASE
EcoRI
EcoRI
TK
pILTK1
Amp r
BamHI
BamHI
EcoRI PARTIAL
DNA POLYMERASE
EcoRI
TK
pGEM3
HindIII
Amp r
PvuII
HINDIII
DNA POLYMERASE
PvuII
PHOSPHATASE
Amp r
T4 DNA LIGASE
BamHI
pILTK5
TK
Amp r

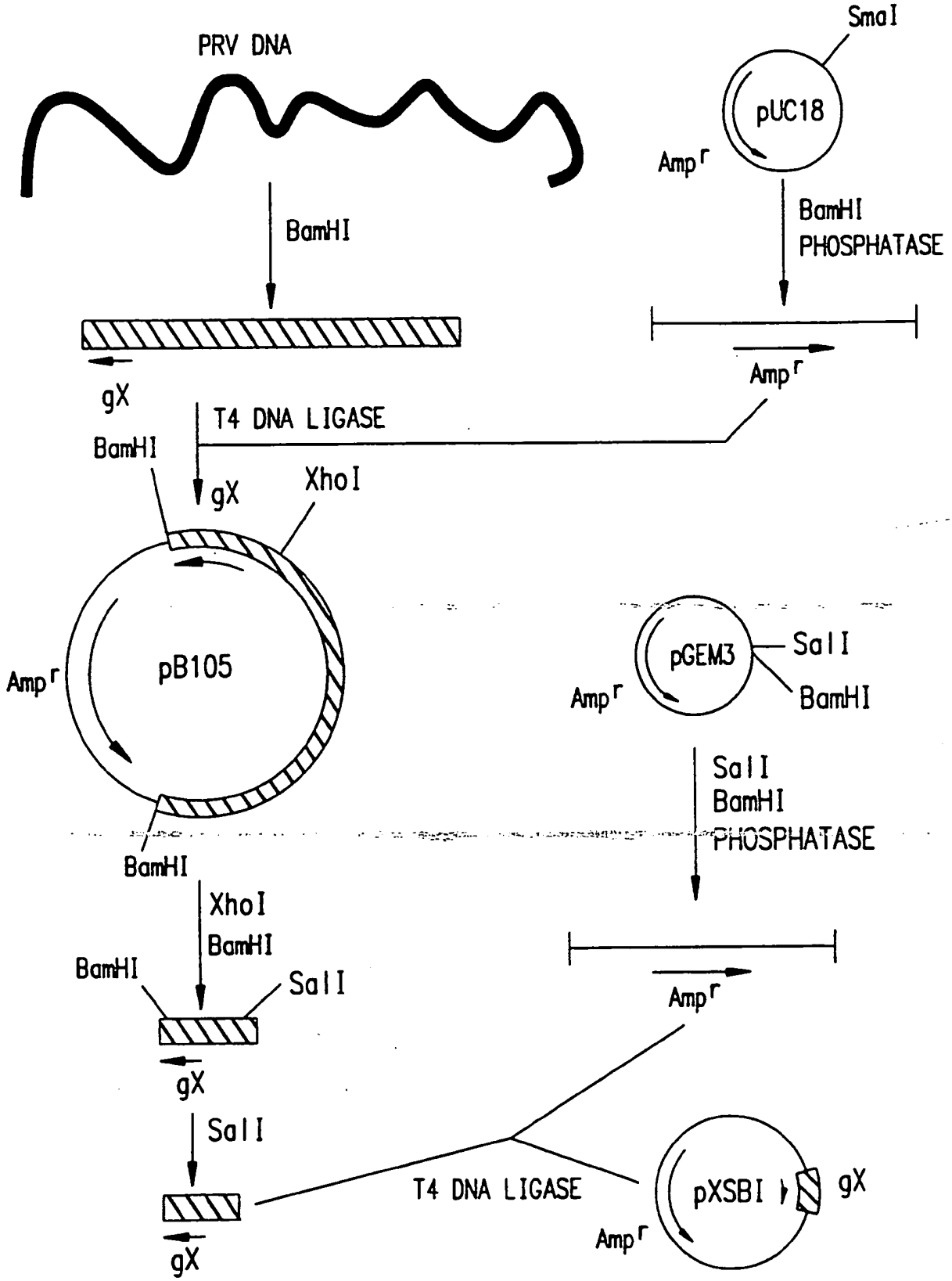

PRV DNA
BamHI
gX
SmaI
pUC18
Amp r
BamHI
PHOSPHATASE
Amp r
T4 DNA LIGASE
BamHI
gX
XhoI
pB105
Amp r
BamHI
XhoI
BamHI
BamHI
SalI
gX
SalI
gX
pGEM3
SalI
BamHI
Amp r
SalI
BamHI
PHOSPHATASE
Amp r
T4 DNA LIGASE
pXSBI
gX
Amp r

FIG. 2

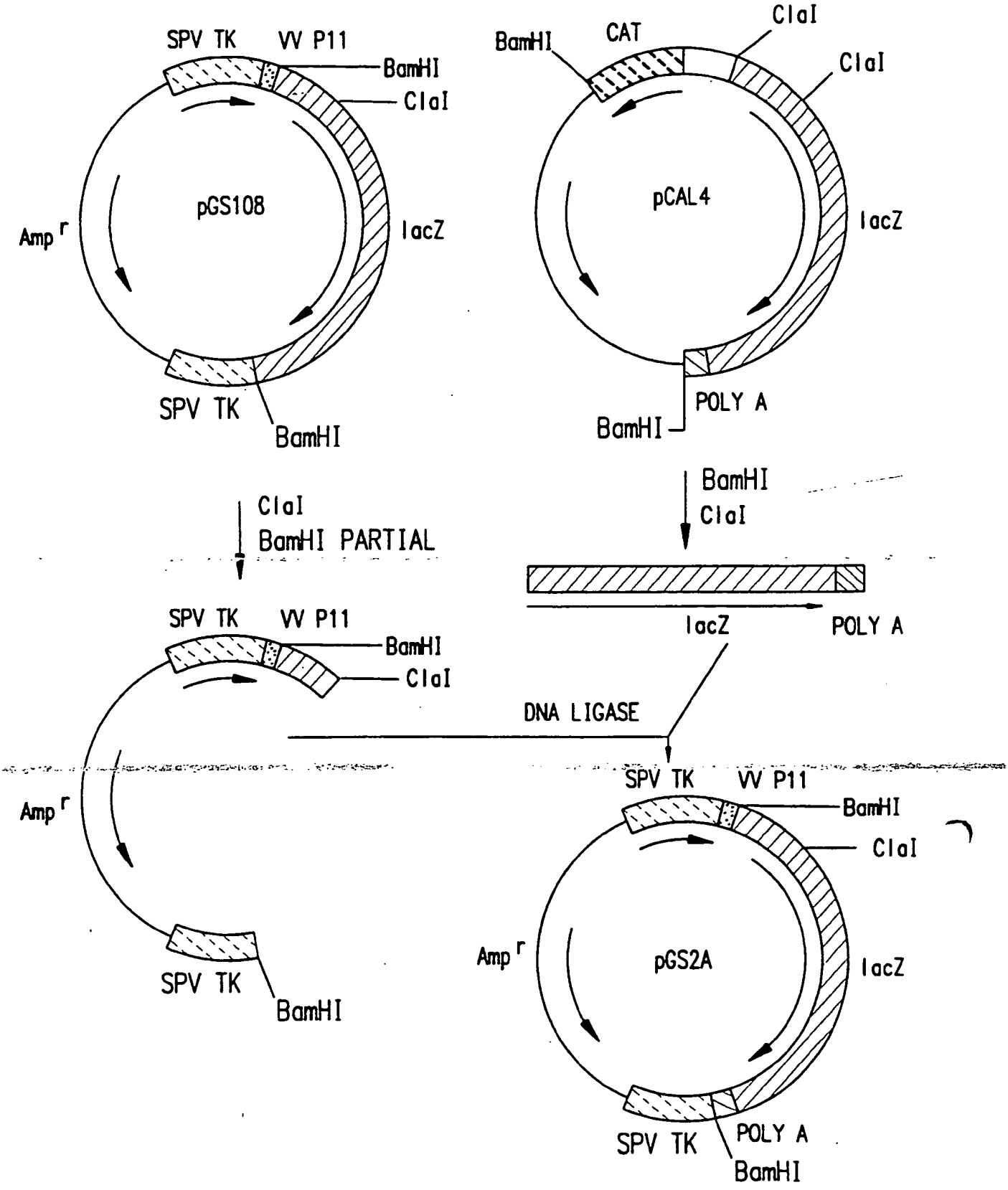

SPV TK
VV P11
BamHI
ClaI
pGS108
Amp r
lacZ
SPV TK
BamHI
BamHI
CAT
ClaI
ClaI
pCAL4
lacZ
POLY A
BamHI
ClaI
BamHI PARTIAL
BamHI
ClaI
lacZ
POLY A
SPV TK
VV P11
BamHI
ClaI
DNA LIGASE
Amp r
SPV TK
BamHI
SPV TK
VV P11
BamHI
ClaI
Amp r
pGS2A
lacZ
SPV TK
POLY A
BamHI

FIG.3

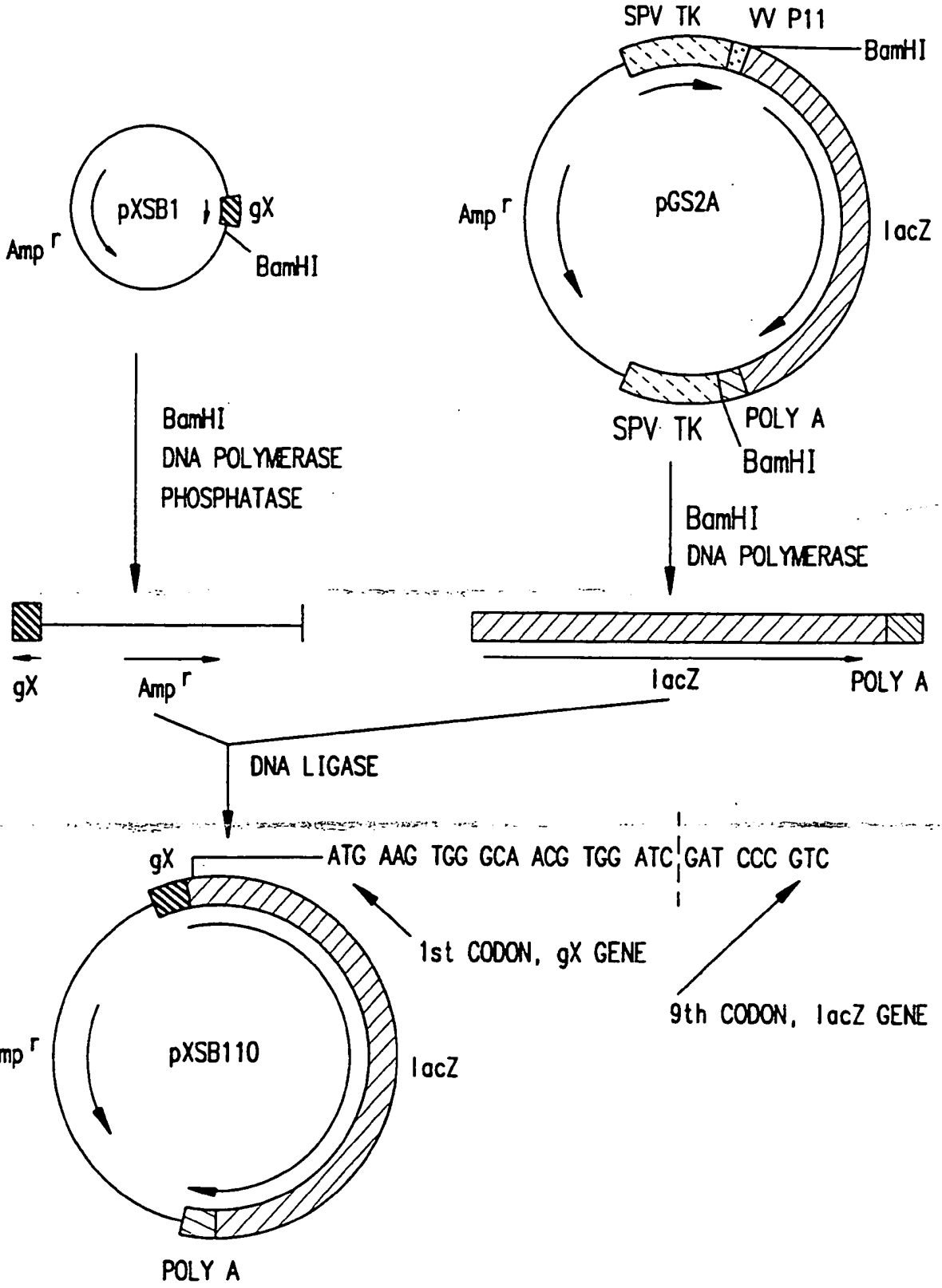

SPV TK
VV P11
BamHI
pXSB1
gX
Amp r
BamHI
pGS2A
Amp r
lacZ
SPV TK
POLY A
BamHI
BamHI
DNA POLYMERASE
PHOSPHATASE
BamHI
DNA POLYMERASE
gX
Amp r
lacZ
POLY A
DNA LIGASE
gX
ATG AAG TGG GCA ACG TGG ATC GAT CCC GTC
1st CODON, gX GENE
9th CODON, lacZ GENE
Amp r
pXSB110
lacZ
POLY A

FIG.4

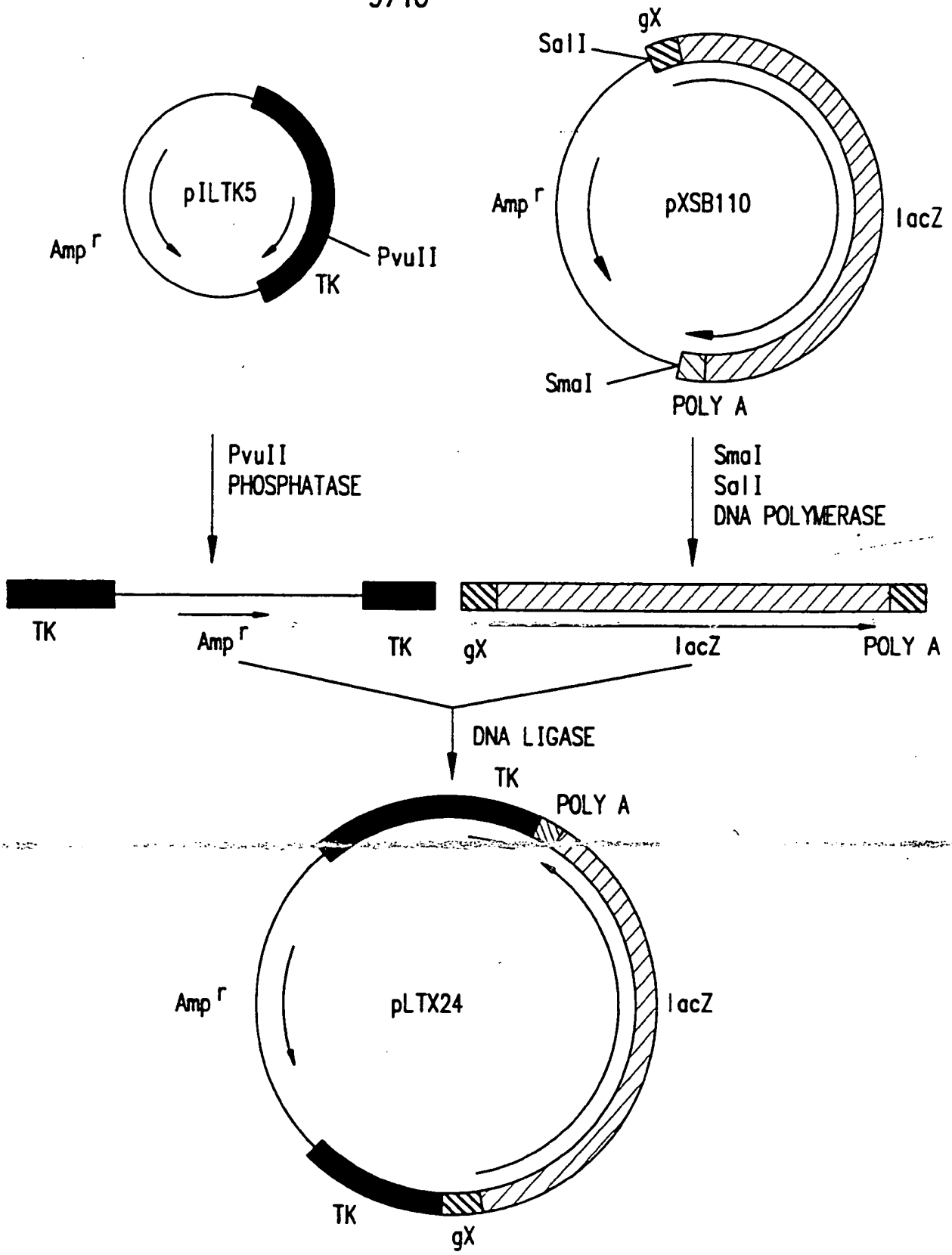

gX
SalI
pILTK5
Amp r
PvuII
TK
Amp r
pXSB110
lacZ
SmaI
POLY A
PvuII
PHOSPHATASE
SmaI
SalI
DNA POLYMERASE
TK
Amp r
TK
gX
lacZ
POLY A
DNA LIGASE
TK
POLY A
Amp r
pLTX24
lacZ
TK
gX

FIG.5

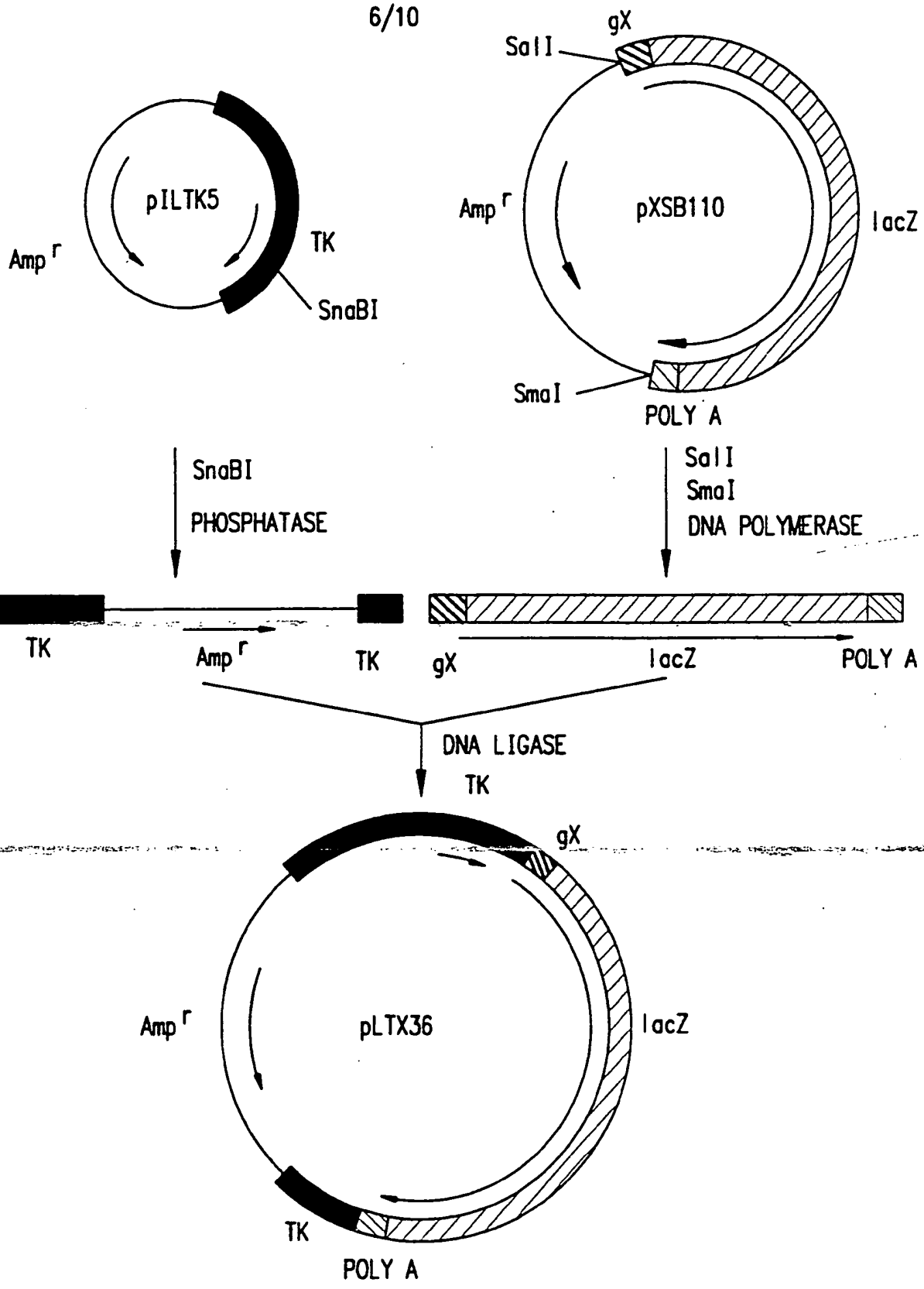

pILTK5
Amp r
TK
SnaBI
pXSB110
Amp r
SalI
gX
lacZ
SmaI
POLY A
SnaBI
PHOSPHATASE
SalI
SmaI
DNA POLYMERASE
TK
Amp r
TK
gX
lacZ
POLY A
DNA LIGASE
TK
gX
Amp r
pLTX36
lacZ
TK
POLY A

FIG.6

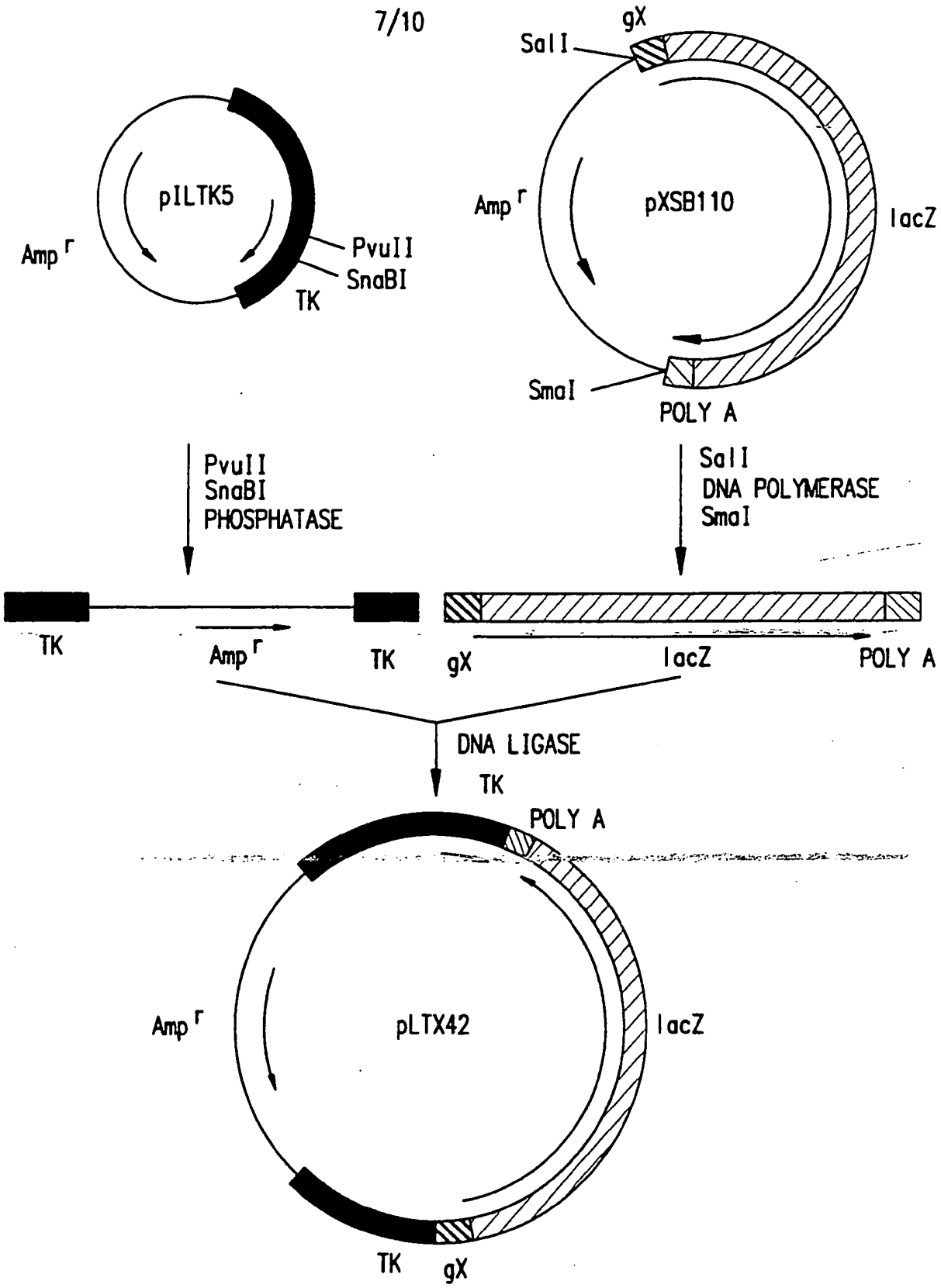

pILTK5
Amp r
PvuII
SnaBI
TK
gX
SalI
pXSB110
Amp r
lacZ
SmaI
POLY A
PvuII
SnaBI
PHOSPHATASE
SalI
DNA POLYMERASE
SmaI
TK
Amp r
TK
gX
lacZ
POLY A
DNA LIGASE
TK
POLY A
pLTX42
Amp r
lacZ
TK
gX

FIG.7

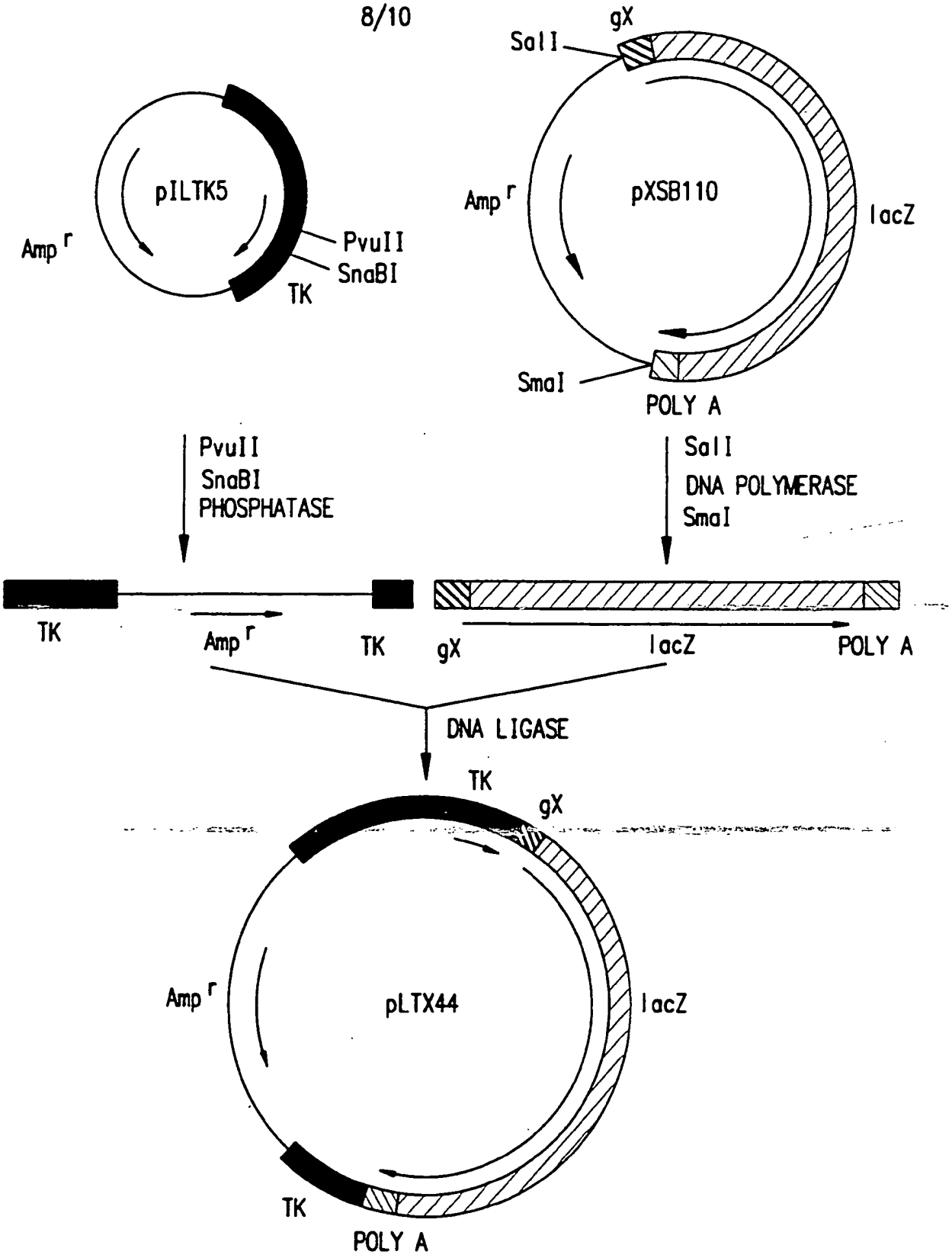

pILTK5
Amp r
PvuII
SnaBI
TK
gX
SalI
pXSB110
Amp r
lacZ
SmaI
POLY A
PvuII
SnaBI
PHOSPHATASE
SalI
DNA POLYMERASE
SmaI
TK
Amp r
TK
gX
lacZ
POLY A
DNA LIGASE
TK
gX
Amp r
pLTX44
lacZ
TK
POLY A

FIG.8

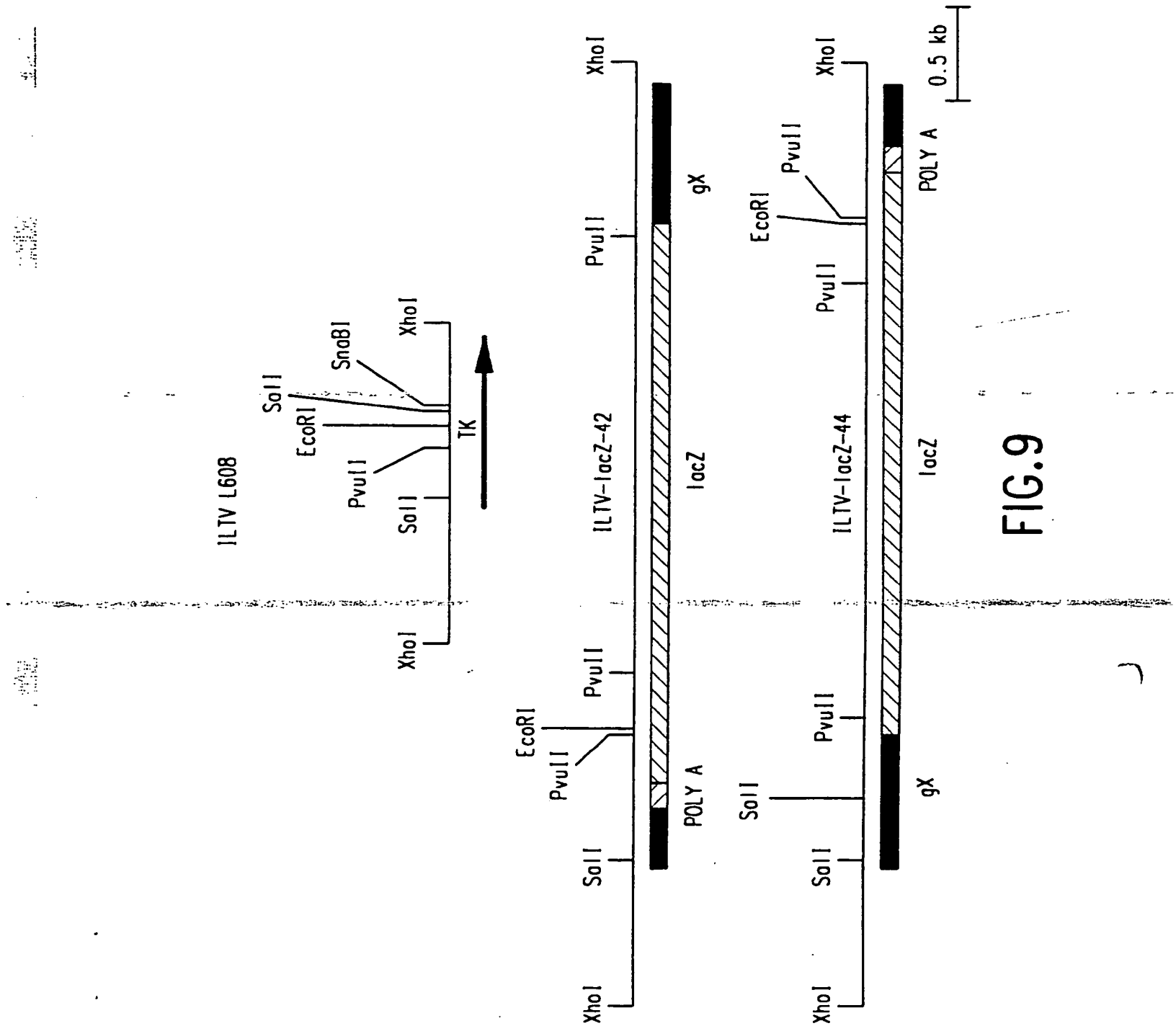

ILTV L608
SalI
EcoRI
PvuII
SalI
XhoI
SnaBI
XhoI
TK
XhoI
PvuII
EcoRI
PvuII
SalI
XhoI
ILTV-lacZ-42
lacZ
gX
POLY A
XhoI
PvuII
EcoRI
PvuII
SalI
XhoI
ILTV-lacZ-44
lacZ
POLY A
gX
0.5 kb

FIG.9

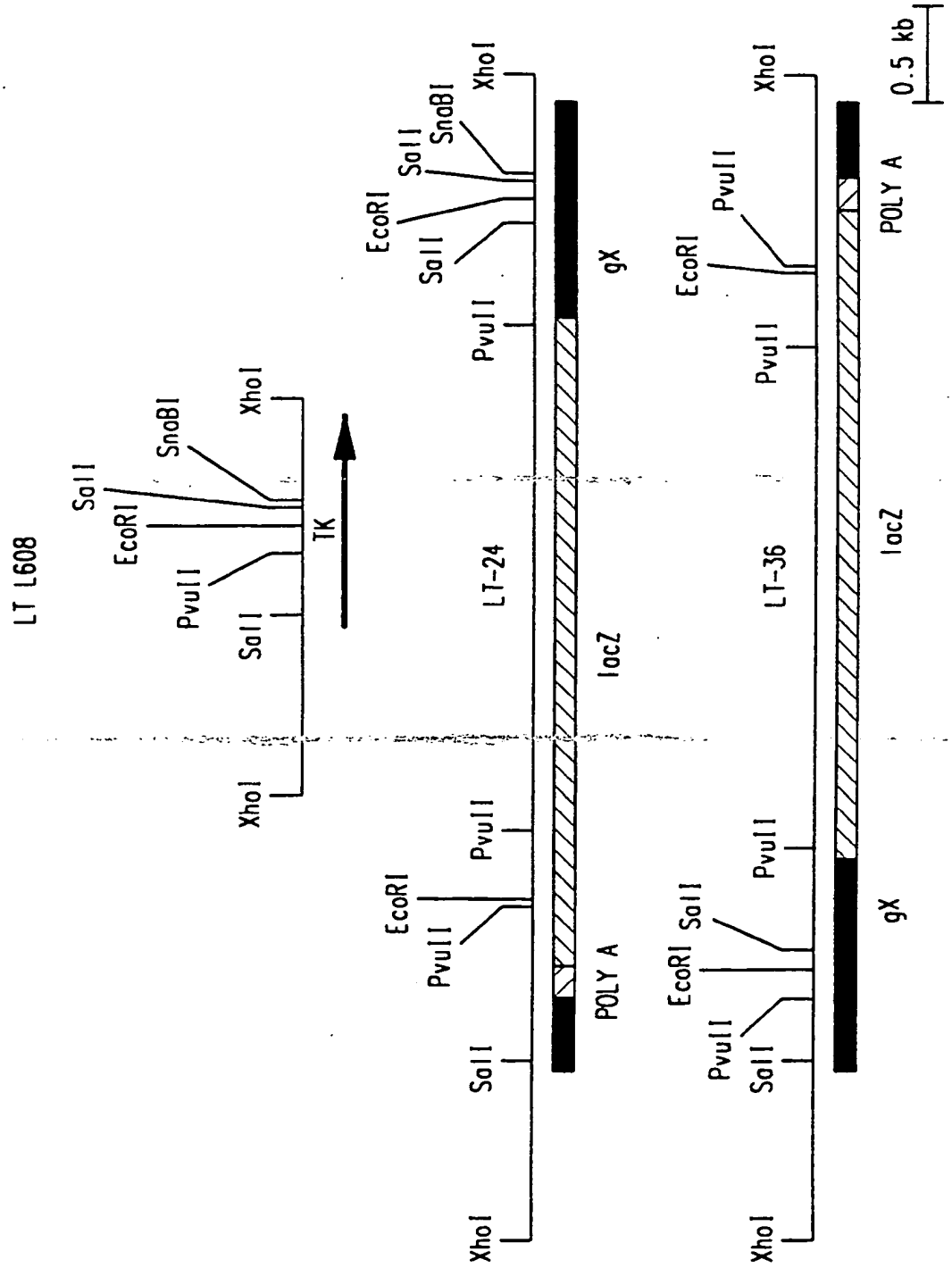

LT L608
XhoI
SalI
PvuII
EcoRI
SnaBI
XhoI
TK
SalI
PvuII
EcoRI
SalI
SnaBI
XhoI
gX
LT-24
lacZ
POLY A
XhoI
SalI
PvuII
EcoRI
PvuII
SalI
XhoI
gX
LT-36
lacZ
POLY A
0.5 kb

FIG.10

INTERNATIONAL SEARCH REPORT

Internatir Application No
PCT/US 95/07862

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C07K14/03 A61K39/245 C12N7/04 //C07K19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,90 02802 (INSTITUTE FOR ANIMAL HEALTH LIMITED) 22 March 1990 see abstract see pages 30-36, especially page 35 example 7	1-20
X	US,A,5 279 965 (KEELER C.L.) 18 January 1994 see abstract see column 5 lines 5-12, lines 40-56 see column 6, line 51 - line 56 see columns 9 and 10 example 2	1,2,4-9, 11-15, 17-20

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 November 1995	0 8. 12. 95
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk	Authorized officer

1

INTERNATIONAL SEARCH REPORT

Internati Application No
PCT/US 95/07862

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 9425 Derwent Publications Ltd., London, GB; Class B04, AN 205018 & JP,A,06 141 853 (ZH. KAGAKU & KESSEI RYOHO KENKYUSHO) , 24 May 1994 see abstract	1,4-8, 11-15, 17-20
X	WO,A,92 03554 (ARTHUR WEBSTER PTY LTD) 5 March 1992 see page 3 - page 7 see page 8, line 1 - line 8 see page 25 - page 27 see page 43 - page 49 see page 53, line 11 - line 23 see page 54	1,2,4-6, 8,11-13, 15,17-20
P,X	VIROLOGY, vol. 209, no. 2, 1 June 1995 pages 304-314, SCHNITZLEIN ET AL. 'Genereation of Thymidine Kinase deficient mutants of infectious laryngotracheitis virus' see the whole document	1-20
P,X	WO,A,95 08622 (SYNTRO CORPORATION) 30 March 1995 see page 3, line 30 - line 38 see page 6 see page 7, line 35 - line 38 see page 8, line 1 - line 5 see page 20 - page 27 see claims 11,15,16,18,20,21,24,50	1,2,4-9, 11-15, 17-20

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat' Application No
PCT/US 95/07862

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9002802	22-03-90	AU-B-	633272	28-01-93
		AU-B-	4214289	02-04-90
		AU-B-	629248	01-10-92
		AU-B-	4325089	02-04-90
		EP-A-	0434721	03-07-91
		EP-A-	0434747	03-07-91
		WO-A-	9002803	22-03-90
		IL-A-	91627	31-08-95
		JP-T-	4501658	26-03-92
		JP-T-	4502852	28-05-92
US-A-5279965	18-01-94	NONE		
WO-A-9203554	05-03-92	NONE		
WO-A-9508622	30-03-95	AU-B-	7838694	10-04-95